Chromogranin A in Tumor and Vascular Biology.

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Chromogranin A (CgA) is a glycoprotein stored in the dense core granules of the adrenal medulla and of many neuroendocrine cells and neurons. This protein is believed to play an intracellular role as a key regulator of secretory granules biogenesis and an extracellular function as a precursor of several regulatory peptides for the endocrine and the metabolic systems. In addition, CgA has been recognized as a useful tissue and serum marker of neuroendocrine tumors and a prognostic indicator in heart failure. A growing body of evidence suggests that CgA is not only an important diagnostic and prognostic marker, but that it could also play important functions in tumor biology and cardiovascular physiology that deserve to be investigated.

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Cell Biology of the Chromaffin Cell
R. Borges & L. Gandía Eds.
Instituto Teófilo Hernando, Spain, 2004

Chromogranin A (CgA) is an acidic glycoprotein belonging to a family of regulated secretory proteins stored in the dense core granules of the adrenal medulla and of many neuroendocrine cells and neurons[1]. This protein was originally identified as the major soluble protein of the secretory granules of chromaffin cells, co-released with catecholamines from stimulated adrenal medulla[2]. CgA was found later to be a member of a larger family of soluble acidic secretory proteins that includes, besides CgA, also CgB, secretogranin (Sg) II (CgC), Sg III (1B1075), Sg IV (HISL-19), Sg V (7B2) and Sg VI (NESP55)[3]. It has been proposed that CgA is a precursor of several biologically active peptides with important roles in the regulation of the endocrine, metabolic and immune systems[4]. In this chapter I will discuss the evidence that suggest that CgA, besides regulating these systems, could also affect the cardiovascular physiology and the tumor biology.

Biochemical properties and detection of cga in tissues and biological fluids. cDNA and protein characterization studies have shown that human CgA is an O-glycosylated, sulphated and phosphorylated protein of 439 residues[4-7]. CgA may undergo pH- and Ca^{2+}-dependent conformational changes causing exposure of hydrophobic residues and formation of dimers or tetramers[8-10]. The C-terminal region is important for dimer-tetramer equilibrium[11,12]. N-terminal fragments (residues 1-78) may also form dimers, at micromolar concentrations, which rapidly dissociate upon dilution[13].

CgA contains a high number of dibasic sites thought to be important for tissue specific proteolytic processing. Moreover, multiple forms having different hydrodynamic sizes of 600 kDa, 100 kDa and 55 kDa have been detected in the serum of cancer patients[14]. The levels of post-translational modification and proteolytic processing may differ from tissue to tissue[15-18]. Thus, from an analytical point of view, CgA is a highly heterogeneous antigen. Since CgA is usually detected in biological fluids and in tissues using different immunological probes (by RIA, ELISA, western blotting, immunofluorescence microscopy, immunohistochemistry, etc.), it is not surprising that different antibodies can detect CgA with different

efficiency. This is an important point to keep in mind when comparing the results obtained with different assays.

Besides chromaffin cells of the adrenal medulla, other cells of the diffuse neuroendocrine system express CgA. For instance, CgA is co-stored with various hormones in the secretory vesicles of cells of the gastrointestinal tract[19], the adeno- and neuro-hypophysis[20], the parathyroid[21], the endocrine pancreas[22], the thyroid C-cells[23], the immune system[24], and the atrial myocardium[25]. In addition, it is a component of dense-core synaptic granules in many areas of the central nervous system[26,27].

CgA is expressed also by many endocrine and neuroendocrine tumors including pheochromocytomas, various carcinoid tumors of the stomach, lung, intestine, prostate and liver, parathyroid carcinoma, medullary thyroid carcinoma, anterior pituitary tumors, pancreaticoduodenal tumors, neural tumors, small cell lung cancer (SCLC) and many others[3,28]. Interestingly, also certain non-neuroendocrine tumors, such as non small cell lung cancer (NSCLC), prostate cancer and breast cancer may undergo neuroendocrine differentiaton and present focal expression of CgA[3].

CgA is exocytotically released in the extracellular environment, and then in circulation, together with co-resident hormones[29]. In normal subjects the circulating levels of CgA are 0.5-2 nM, depending on the immunoassay used. Circulating CgA can increase several folds in patients with pheochromocytomas and up 0.1-1 μM in patients with carcinoid tumors[30,31]. Many other neuroendocrine tumors can release CgA in circulation, with important diagnostic and prognostic implications[3]. However, CgA levels can increase also in patients with renal failure, hepatic failure, cardiac arrest, heart failure or essential hypertension[3].

Biological activities of CgA. It has been proposed that CgA plays an important role in secretory vesicle biogenesis and hormone packaging[32]. Besides these intracellular functions it is believed that CgA can also play many extracellular functions. The presence of several dibasic sites potentially cleaved by proteases and the observation of tissue-specific proteolytic processing led to the hypothesis that CgA is a precursor of various biologically active

peptides[4,33,34] with endocrine, paracrine and autocrine functions. For instance, CgA residues 248-293 were found to be homologous to pancreastatin, a pancreatic peptide that regulates glucose and lipid metabolism[34], whereas catestatin, a peptide corresponding to residues 344-364 of bovine CgA, inhibits secretion of catecholamines from catecholaminergic cells[35]. Fragments corresponding to aminoacids 1-76 and 1-113, named vasostatin-1 and vasostatin-2, suppress vasoconstriction in isolated blood vessels[36,38]. Vasostatin-1 can also inhibit parathyroid hormone secretion[39], is neurotoxic in neuronal/microglial cell cultures[40], and induces antibacterial and anti-fungal effects[41]. The structural determinants of these activities are located in different regions of the N-terminal domain. For example, peptide 1-40, containing the Cys_{17}-Cys_{38} disulfide bridge, induces vasodilator effects and inhibits parathormone secretion, whereas peptide 47-60 can kill a variety of filamentous fungi[41,42].

In the last years we have found that CgA and vasostatin-1 can modulate, in an indirect manner, fibroblast- and smooth muscle cell-adhesion[43,44]. More recently, we have found that CgA can also affect cell-cell adhesion and permeability of endothelial monolayers[45]. Other studies showed that CgA, at nanomolar concentration, may increase deposition of basement membrane components, such as collagen type IV, laminin and perlecan by mammary epithelial cells, and alter ductal morphogenesis *in vitro*[46], suggesting a role of CgA in cell adhesion and tissue morphogenesis.

CgA and cell adhesion. Solid-phase bound CgA exerts anti-adhesive effects in fibroblast adhesion assays, whereas the N-terminal fragment 1-78 exerts pro-adhesive effects[43]. Proteolytic processing of natural CgA with plasmin decreases its anti-adhesive activity and induces pro-adhesive effects in fibronectin or serum dependent fibroblast adhesion assays[47]. It would appear, therefore, that this protein might work on one hand as a negative modulator of fibroblast adhesion and on the other hand as a precursor of positive modulators. Given the well-recognized importance of fibroblasts and plasminogen activation in tissue invasion, remodeling and repair[53-56], the interplay between CgA and plasminogen/plasmin system could provide a novel mechanism for regulating fibroblast adhesion and function in tumors.

CgA is present in neuroendocrine secretory vesicles at very high concentrations, approaching millimolar levels[50]. Elevated levels of CgA (up to 100-1000 nM) have been detected in the blood of patients with different neuroendocrine tumors[51,52]. Given that CgA and CgA1-78 affect fibroblast adhesion at 7-70 nM and 30-300 nM, respectively[47], it is possible that CgA reaches sufficient levels in tumors to affect stromal fibroblasts. Further work is necessary to assess how CgA is processed within the tumor, e.g. by plasmin, as this could have important effects on its positive or negative effect on stromal fibroblasts.

The results of structure-function studies suggest that the region 47-64 (RILSILRHQNLLKELQDL) is critical for the pro-adhesive activity[44]. This region is 100%-conserved in human, porcine, bovine, equine, and mouse CgA and is 89%-conserved in frog CgA[5,48], pointing to a functional importance. NMR analysis of peptide 47-66[42] suggests that the region 47-51 forms a short hydrophobic helix, followed by an amphipathic helix (residues 53-66). Interestingly, the 47-64 region, besides containing a pro-adhesive site also contains a Ca^{2+}-dependent calmodulin binding site[49].

The receptors or the molecular targets of CgA in cell adhesion are unknown. Analysis of the primary structure revealed that an RGD integrin binding motifs, often present in proteins of the extracellular matrix, is present at residues 43-45. However, a recombinant RGE-CgA(7-439) mutant induced anti-adhesive and pro-adhesive effects after tryptic digestion, as the wild type recombinant RGD-CgA(7-439) [43]. Thus, molecular targets different from RGD-binding receptors must be sought.

CgA and the cardiovascular system. A growing body of evidence suggests that CgA could play a role in the regulation of the cardiovascular system. For instance, the inhibition of catecholamines secretion from catecholaminergic cells by catestatin[35], the vasoinhibitory activity of vasostatins on isolated vessels[38,57], and their negative inotropic activity on isolated working heart[58] suggest that CgA is a precursor of cardiovascular regulatory peptides. Moreover, we have recently reported that CgA can regulate the endothelial cell shape and barrier function[59]. Using mice bearing subcutaneous tumors

genetically engineered to secrete CgA in circulation we have found that increased blood levels of this protein prevent vascular leakage induced by TNF in the liver venous system[45]. Structure-activity studies, carried out with CgA fragments administered to normal mice, showed that an active site is located within the vasostatin-1 domain. Studies of the mechanism of action showed that CgA inhibits TNF-induced VE-cadherin down-regulation and barrier alteration of cultured endothelial cells. These findings suggest that circulating CgA could contribute to regulate the endothelial barrier function and to protect vessels against TNF-induced plasma leakage in pathological conditions characterized by increased production of TNF and CgA.

Interestingly, circulating CgA is increased in patients with chronic heart failure (CHF) depending on the clinical severity of the syndrome[60]. Circulating CgA level is an independent predictor for mortality in these patients. While little or no correlation was observed with adrenaline, noradrenaline, atrial natriuretic factor, aldosterone and plasma renin activity, a good correlation was found between CgA and soluble TNF p55 and p75 receptors[61]. These results point to a regulatory link between cells that secrete CgA and cells that release sTNF-Rs. Although the clinical significance of TNF production in heart failure patients remains uncertain, its ability to induce cachexia, left ventricular dysfunction and pulmonary edema, suggests that TNF, in concert with other inflammatory cytokines, could play a pathogenetic role[62-66]. Considering the inhibitory effect of CgA on TNF-induced vascular leakage observed in mice[45] it is possible that regulated secretion of CgA, in concert with TNF soluble receptors, contributes to reduce the potentially dangerous effects of pathological levels of TNF on the vascular function.

Persistent activation of the neuroendocrine system, particularly the adrenergic and the renin-angiotensin systems, is maladaptive in heart failure[67]. Given that catestatin is a potent non-competitive inhibitor of nicotinic cholinergic receptor mediated catecholamine release[35] and that vasostatin-1 can inhibit noradrenaline-induced vascular tone[38] as well as the positive inotropism induced by isoproterenol in experimental models[58], it is possible that secretion of CgA contributes to counteract the excessive activation of the neuroendocrine system. However, further work is necessary to assess

whether proteolytic processing of CgA occurs in heart failure, and to measure local and systemic concentration of CgA peptides.

CgA in tumor biology. CgA is abnormally expressed by various neuroendocrine tumors and is released in high amounts in the blood stream[28,30,68-71]. Detection of CgA in biological fluids and in tumor tissues has proven useful for diagnosis and for monitoring tumor progression/regression after therapy[3]. However, little is known on the effect of excessive production of CgA on the tumor growth and behavior.

To investigate the effect of CgA secretion on tumor growth we have transfected mouse RMA lymphoma and TS/A adenocarcinoma cells (CgA negative) with the cDNA encoding CgA and studied their proliferation and tumorigenicity *in vitro* and *in vivo*. The most striking observation was that CgA expression was associated with a decreased tumorigenicity in mice. Moreover, CgA production was associated with increased tumor necrosis and multi-nodular growth pattern. Studies on the mechanisms of action showed that CgA expression does not affect the *in vitro* proliferation index of tumor cells, whereas it affects the *in vivo* growth[72]. This suggests that the effect is indirect and host-mediated. One possibility is that CgA affects the complex interplay between neoplastic cells and tumor stroma, which is critical for tumor growth. Given that CgA and its N-terminal fragments can inhibit vascular permeability[45], it is possible that CgA inhibits tumor growth by affecting the vascular compartment of the tumor, for instance, by decreasing the transport of macromolecules critical for tumor cell proliferation across the endothelial barrier. In addition, considering the effect of CgA on fibroblast adhesion, it is also possible that CgA affects the tumor architecture by modulating the physiology of stromal fibroblasts within the tumor, which in turn are important for the production of extracellular matrix proteins and stroma formation. These mechanisms are not mutually exclusive.

CONCLUSIONS

Although the extracellular role of CgA remains rather obscure, a growing body of evidence suggests that abnormal secretion of CgA, e.g. by neuroendocrine tumors or by the (neuro)endocrine system in

heart failure or other pathological conditions, is not simply an epiphenomenon of cell secretory activity, but that it could play important functions in tumor and vascular biology that deserve to be investigated.

ACKNOLEDGEMENTS

This work was supported by the Associazione Italiana Ricerca sul Cancro (AIRC).

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